Probing the cytotoxic effect of MT1-MMP targeted anticancer prodrug

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The major limitation of the currently used anticancer agents is narrow therapeutic indices due to the lack of their tumor-selective targeting and consequent off-target toxicity towards normal cells. Accordingly, improving the therapeutic indices, tumor selectivity and response of these agents are paramount. One class of anticancer chemotherapeutics is the vascular disrupting agents (VDAs). Disruption of tumor vasculature is efficient approach to induce blood vessel shutdown with consequent tumor shrinkage and necrosis. The clinical value of several VDAs is compromised by low therapeutic indices and systemic toxicities. One approach is to develop "Tumor-activated prodrugs, TAPs" of these potent VDAs by exploiting specific enzymes synthesized by tumor cells such as matrix metallic-proteinases (MMPs). This study describing the design and the cytotoxicity profile of novel MT1-MMP targeted peptide conjugate of VDA (ICT2588), which is designed to remain nontoxic until metabolized selectively in tumor microenvironment by MT1-MMP. Lead compound is composed of potent VDA of colchicine-binding agent (MB2) linked to specific peptide conjugate rationalized to be selectively metabolized by MT1-MMP which is highly expressed in different cancer cell lines. ICT2588 showed a remarkable cytotoxic effect against the MT1-MMP-positive HT1080 cancer cell line, effectively metabolized by different tumor xenografts but not by normal tissues. Metabolism is further confirmed by blocking the activity MT1-MMP. MT1-MMP is unique tumor feature and can be utilized to activate masked anticancer prodrugs selectively in tumor microenvironment.

Keywords: Membrane-type matrix metalloproteinase, tumor-activated prodrugs

1. Introduction

Cancer develops from gradual accumulation of various genetic alterations in the cell over time which ultimately result in uncontrollable cell growth and tumor development with cells able to metastasize [1]. Cancer development is a complex process dictates from at least six altered cellular features include; evasion of apoptosis, limitless replication potential, self-sufficiency in growth signals and insensitivity to growth-inhibitory signals, sustained tissue invasion, metastasis and angiogenesis. Healthy mammalian cells hold an intrinsic program to limit their replicative potential and division (senescence) once the required and the maximum cell number is achieved. This replicative process is normally controlled via telomeres in normal cells [1]. In cancer cells, the length of telomere is maintained at a length above a critical threshold mainly through the reactivation and the ectopic expression of "telomerase" which leads to an override of replicative control [2]. Alternatively, the equilibrium between cell death and differentiation is maintained by several external signals which stimulate the cells to undergo replication or to die. Such balance is dyes-regulated in cancer cells and results in uncontrolled cellular proliferations, ineffective apoptosis and reduced dependency on exogenous growth factors [1]. Cancer cells are also "self-sufficient" due to some alterations in extracellular growth signals, and insensitive to growth inhibitory signals such as transforming growth factor-β (TGFβ) which is a potent cell-quiescence inducer. In normal circumstances, uncontrolled cell divisions are tightly regulated by apoptosis (programmed cell death) in which cytoskeleton are destroyed, nucleus is fragmented, chromosomes are deteriorated and cell membrane is damaged [1]. Alteration in apoptotic protein expression, mutation or loss of function of p53 (guardian of the genome), are the common mechanisms through which cancer cells evade programmed cell death [1]. Moreover, tumor progression and angiogenesis are closely linked with each other as tumor growth and expansion necessitate more nutrients and oxygen. It is well known that tumors do not growth more than 1-2 mm^2 unless there is a sufficient supply of nutrient and oxygen [3]. Angiogenesis is a dynamic process involves different cells and pro-antigenic factors such as vascular endothelial growth factors (VEGF) and fibroblast growth factor (FGF) [4]. Tumor cells activate the process of angiogenesis by suppressing the anti-angiogenic and enhancing the activity of pro-antigenic factors. Finally, Tumor cells are able to invade the adjacent tissues and travel to distant places at which the nutrients and space are not limiting and they may succeed to form new colonies. These distal deposits of cancer cells are the major cause of cancer deaths.

The clinical use of classical cytotoxic drugs is limited by their acute toxicity to the normally divided cells which leads to wide range of side effects such as; vomiting, nausea, myelosuppression, hair loss and gastrointestinal abnormalities. Most the currently used anticancer drugs have a very narrow therapeutic indices based on their high concentrations required at the tumor site and their high rate of systemic toxicities [5]. Moreover, resistance of cancer cells to wide variety of chemotherapeutic agents is another looming concern. Some treatment modalities are developed in order to overcome anticancer drug resistance such as the concomitant use of vascular disrupting agents (VDAs) and anti-angiogenic drugs. However, acquired drug resistance is still a problem for a wide range of cytotoxic agents.

Tumor targeted therapeutics

The main duty of today's research activities is to develop novel anticancer drugs with enhanced tumor selectivity and wider therapeutic indices. One approach is utilizing the specific effect of tumor phenotype such as; the altered extracellular domain of cell surface receptor(s), increased extracellular matrix (ECM) degradation and some other unique features of tumor microenvironment. The success of this approach was the significant results achieved by the small molecule inhibitors of epidermal growth factor receptors "Gefitinib and Erlotinib" [6]. Despite the significant achievements of these small molecule inhibitors, discovery and validation of their critical targets are sophisticated due to the diversity of genetic alterations and instability of tumor cells.

Tumor-activated prodrugs

An alternative approach is the development of tumor-activated pro-drugs (TAPs) which are non-toxic compounds, chemically, enzymatically or spontaneously activated in tumor tissues after their administration to form the pharmacologically active entities. In this context, unique phenotypic characteristics available at the tumor soil (microenvironment) are utilized to selectively activate a masked and inactive, anticancer pro-drug. Accordingly, the therapeutic entity is delivered to their intended site. TAPs should basically undergo unique metabolism in tumor cells to produce the active therapeutic agent.

Regardless of the mechanisms of their tumor-selective activation, TAPs are generally composed of three domains; effector (responsible for anticancer effect), linker (masks drug activity) and trigger (activity control), as shown in Figure 1 [7].

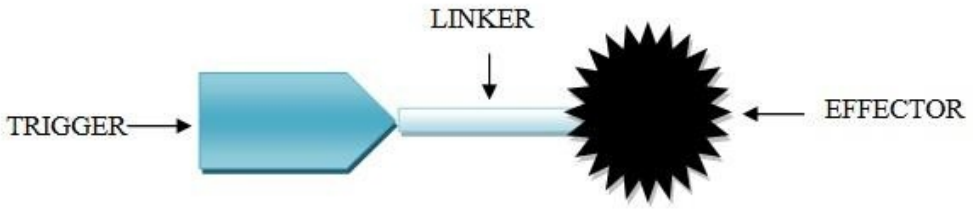

Figure 1: Composition and design of TAPs

TAPs activated by excreted tumor-specific enzymes

Enzyme-activated TAPs are potent and non-toxic pro-drugs developed on the basis of enzymatic differences between tumor and normal tissues. The therapeutic entity "warhead" of TAPs is masked until selectively activated by a unique enzyme(s) in tumor microenvironment (Figure 2) [7].

The initial step in the development of TAPs activated by specific-tumor enzymes is probing the ideal properties of tumor enzyme eligible for targeting by TAPs. Such properties are; the high ability and affinity to selectively activate the pro-drug, undetectable in patient serum, no or little expression and lack of enzymatic activity in normal cells and tissues, significant expression in tumor cells and microenvironment, and involved in tumor progression and development. All criteria are assembled in one enzyme class involved in tumor progression known as "matrix metalloproteinase, MMPs", which is the main focus of this study.

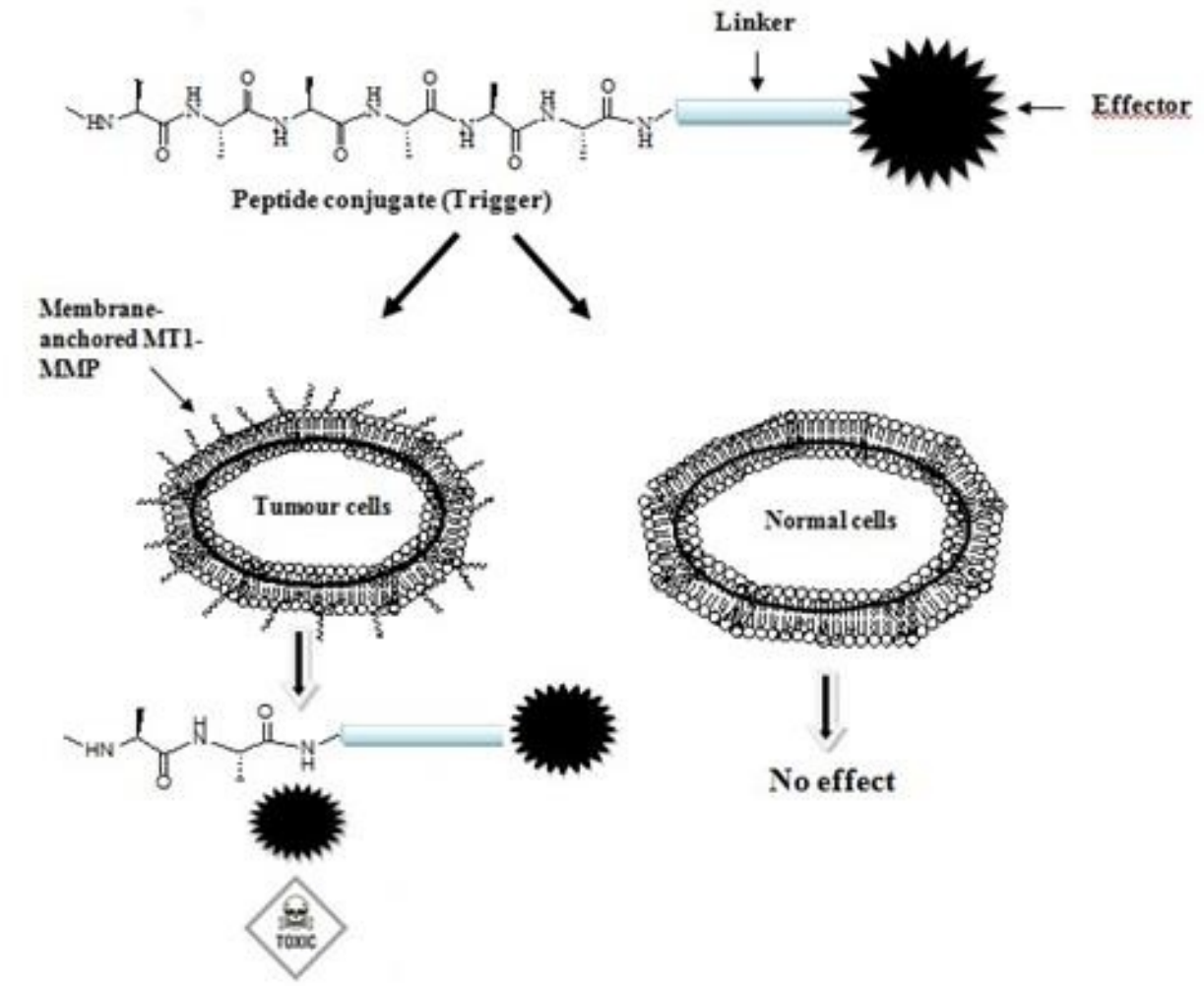

Figure 2. Schematic representation of tumor MMPs-activated prodrugs (TAPs). Three domains are the main composition of the prodrug; effector, linker and trigger. The prodrug remains inactive until activation of trigger by the MMP(s) of choice selectively in the tumour tissues, causing the release of the active drug entity and subsequent cytotoxic effect.

Matrix metalloproteinase (MMPs)

Matrix metalloproteinase (MMPs), also called matrix ins, are a family of highly homologous zinc-dependent proteinases responsible on ECM degradation. There are 24 different members of human MMPs which can be classified either on the basis of their substrate specificity and sequence similarity into five distinct groups; gelatinases (MMP-2, MMP-9), collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10, -11), matrilysins and membrane-type MMPs. Membrane-type MMPs undergo intracellular activation before being covalently linked to the cell membrane via its transmembrane and cytoplasmic domains.[8].

The proteolytic activity of MMPs is firmly controlled by endogenous low molecular weight inhibitors known as "tissue-inhibitor MPs" or (TIMPs-1, 2, 3 and 4) which irreversibly bind and inhibit the active form of MMPs in stoichiometric fashion of 1:1. In comparison to the number of MMPs, the number of TIMPs is basically limited and the balance between MMPs and their inhibitors must be tightly controlled and exist only at the stages that necessitate the process of ECM-breakdown such as physiological developmental stages, as shown in Table 1 [9].

Table 1 Classification of MMPs family

MMP	Structural class	Common name of the protein	MMP	Structural class	Common name of the protein
MMP-1	Hemopexin domain	Interstitial collagenase,	**MMP-19**	Hemopexin domain	RASI-1
MMP-2	Gelatin-binding	Gelatinase A	**MMP-20**	Hemopexin domain	Enamelysin
MMP-3	Simple hemopexin domain	Stromelysin-1, Proteoglycanase	**MMP-21**	Vitronectin-like insert	Homologue of *Xenopus* MMP
MMP-7	Minimal domain	Matrilysin, Matrin	**MMP-22**	Hemopexin domain	CMMP (Chicken)
MMP-8	Hemopexin domain	Collagenase-2	**MMP-23**	Type II transmembr	Femalysin
MMP-9	Gelatin-binding	Gelatinase B	**MMP-24**	Transmembrane domain	MT5-MMP
MMP-10	Hemopexin domain	Stromelysin-2	**MMP-25**	GPI-linked	MT6-MMP
MMP-11	Furin-activated	Stromelysin-3	**MMP-26**	Minimal domain	Matrilysin-2
MMP-12	Hemopexin domain	Metalloelastase			
MMP-13	Hemopexin domain	Collagenase-3			
MMP-14	Transmembrane domain	MT1-MMP			
MMP-15	Transmembrane domain	MT2-MMP			
MMP-16	Transmembrane domain	MT3-MMP			
MMP-17	GPI-linked	MT4-MMP			

MMPs in human cancer

Expression and activity of various MMPs have been found to be increased in the vast majority of human cancers which indicate the invasive and metastatic features, and in general, shortened survival [10]. MMPs can be expressed by cancer cells themselves or the surrounding stromal cells to remodel the surrounding basement membrane and ECM and thus release its embedded growth factors that are required for tumor growth, angiogenesis, invasion and metastasis [11]. MMPs are also capable to cleave a wide range of tumorigenic non-matrix substrates such as: cleavage and activation of growth factors (e.g, TGF-α), suppression of apoptosis (Fas-ligand) [12], activation of cell division (FGF, TGF-β), degradation of chemokines, alteration of several signaling pathways and release of antigenic factors (mainly VEGF) [13]. Furthermore, MMPs are essential for clearing the surrounding EMC and thereby paving the way in front of cancer cells and enhance their metastasis. Inhibitors of MMPs have been developed as psuedopeptides mimicking MMP substrates, small molecule inhibitors of the catalytic zinc and agents that block the synthesis of MMPs (antisense mRNA or by targeting mRNA with ribozymes) [14]. However, their initial clinical trials were disappointing and ended with limited anticancer effects. Alternatively, increased MMPs activity in tumors can be exploited to selectively activate anticancer prodrugs. MMPs demonstrate elevated activity in tumor micro-region, no or little activity in normal cells and capable to specifically cleave small peptide sequences [15]. Initially, it has been thought that the expression of MMPs by the host stromal cells would be a problem for the development for MMPs-activated pro-drugs. In fact, stromal cells that are adjacent to tumor tissues and produce MMPs are known to potentiate malignant behavior. In addition, expression of MMPs by stromal cells elevates the level of target for TAP activation. As such, the expression of MMPs by stromal cells is considered as a positive factor for the success of MMPs-activated anticancer pro-drugs [15].

Secreted MMPs-tumor activated pro-drugs

One approach was the incorporation of water-soluble maleimide derivative of doxorubicin into MMP2-cleavable peptide sequence. Purified MMP-2 and MMP-2 tissue homogenate demonstrated the efficiency of doxorubicin release and studies used A357 melanoma showed superior activity and higher tolerated-dose than that of doxorubicin alone [16]. In another approach, an anthraquinonoid topo-isomerase inhibitor (NU: UB31) was incorporated into the C-terminus of MMP-9-heptapeptide conjugate and the N-terminus of the peptide are “end-capped” with fluorescein is thiocyanate (FITC) to prevent any unrequired exopeptidase degradation. The end product of this approach was “EV1-FITC-prodrug”. There was, however, one experienced downside resulted from the lack of TAP selectivity towards MMP-9 and thus, profound level of EV1-activation in normal tissue homogenates was detected [17]. According to these evidences, the downside of targeting the secreted-MMPs is their innate ability to diffuse small distances from tumor cells which may result in drug activation away from the tumor tissues or leaking into the circulation. Since membrane-type MMPs are localized on the cellular surface of tumor and stromal cells and thereby the area of pro-drug activation is more controlled, development of TAPs towards

the “membrane-type” MMPs would result in local and more selective pro-drug activation than the secreted MMPs-targeted TAPs.

Membrane-type matrix metalloproteinase (MT-MMP)

MT1-MMP contains all protein domains of MMPs alongside its unique structural domains which include: a furin-recognition domain (~11 amino acids) located between the zinc-containing catalytic and the pro-peptide domain which is a site for convertase-dependent activation of zymogen to the active MT1-MMP. MT1-MMP also contains a distinctive, hydrophobic C-terminus stretch of about 20 amino acids representing the transmembrane domain of MT1-MMP followed by a short cytoplasmic tail, as shown in Figure 3 [18].

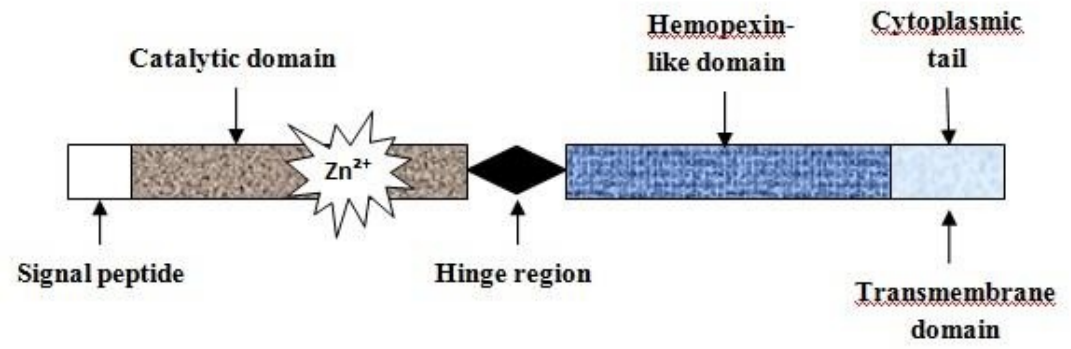

Figure 3. Structure and domain arrangements of MT1-MMP

Roles of MT1-MMP in cancer

MT1-MMP has a broad spectrum of activity against several ECM macromolecules such as; type I and II collagen [19], fibronectin, vitronectin, fibrin, proteoglycans and laminins-5 (leads to stimulation of EGF receptors and enhanced cell motility), cell surface proteins such as CD44 in association with A Disintegrin and A Metalloprotease domain (ADAM-like proteases) , syndecan-1 (result in altered integrins signaling and enhanced cell motility), αv-integrins, and collagen phagocytosis, activation of some oncogenic signaling pathways such as extracellular signal-regulated protein kinase (ERK), and mitogen-activated protein kinase (MAPK) [20].

MT1-MMP is required, or at least involved, in tumor angiogenesis, processing and shedding of semaphoring-4D, interaction with αvβ3 integrin, and transcriptional activations of VEGF.

MT1-MMP tumor-activated prodrugs

MT1-MMP-activated liposomal drug delivery is the only approach that has harnessed MT1-MMP to selectively release liposomes-entrapped lipophilic derivative of novel anti-angiogenic agent. However, delivery of liposomes to the poorly vascularized tumor tissues is difficult and thus, TAPs derived from peptide-drug conjugates are still more attractive approach for the development of anticancer pro-drugs [21].

In order to overcome the "warhead" activation outside tumor tissues and to avoid difficulty of liposomal delivery, the Institute of Cancer Therapeutics, University of Bradford, has developed a novel TAP to selectively target the MT1-MMP, known as ICT2588. Unlike the MMPs-targeted strategies, the rationale of this approach is based not only on the fact that MT1-MMP is vital and unique tumor feature, but also on the basis of its cell surface localization. In this context, the active "warhead" will be selectively released in tumor tissues.

Chemistry and design of ICT2588

General sequence substrate of MMPs has been identified to contain: a Serine (Ser) moiety at the P^1 position with Leucine/Methionine as a $P^{1\prime}$ residue and a Proline (Pro) in the P^3 position [22]. Figure 4a. These substrates are collagen-like, non-selective and can be recognized by many MMPs active pockets. The symbol (P) represents the position of amino acid from the scissile bond, P′ residues are those from the scissile bond to the carboxyl terminus, whilst P residues are from the scissile bond to the amino terminus of the peptide sequence (Figure 4a).

In contrast to MMPs-substrates, MT1-MMP was found to preferentially cleave linear substrates that have Ser instead of Pro moiety in the P^3 position and Arg instead of glutamic acid in the P^4 position [23]. The N-terminus of the peptide was 'capped' with fluorescein is thiocyanate (FITC), to produce a TAP, EV1–FITC. The presence of hydrophobic alkyl chain at the $P^{1'}$ position, such as Phe, and the Arg residue in the P^4 position were assumed to enhance the selectivity of these substrates towards MT1-MMP (Figure 4a). The active warhead "effector" of ICT2588 is a colchicines derivative VDA, azademethyl-colchicine also called MB2 (Figure 4b). The rationale of using MB2 is based on the fact that MB2 has demonstrated more potent vascular disrupting effect than colchicines (Dr. J. Gill, personal communications) (Figure 4c). Furthermore, a previous work at the Institute of Cancer Therapeutics showed that ICT2588 is cleaved by MT1-MMP at the Gly-Hof (P^1-$P^{1'}$)-scissile bond. (Figure 4d). In comparison to MMPs-activated prodrugs, MMP-9 was not able to cleave the ICT2588 but MMP-2 was able to cleave the ICT2588 between Hof-Tyr peptide bond. Accordingly, selectivity of ICT2588 toward MT1-MMP could be enhanced by amino acid modification at the $P^{2'}$ position where polar amino acids such as Arg, Glu, Tyr or Lys increase the selectivity of peptide conjugates towards MT1-MMP [24].

Disruption of established tumor vasculature is powerful and attractive chemotherapeutic approach to starve solid tumors of oxygen and nutrients and consequently induce tumor shrinkage and necrosis [25]. However, progression of several VDAs through the clinical trials is compromised by their off-target cardio toxic effects, mainly arrhythmias and cardiac ischemia. Accordingly, these agents show narrow therapeutic indices which necessitate its selective targeting to the tumor tissues.

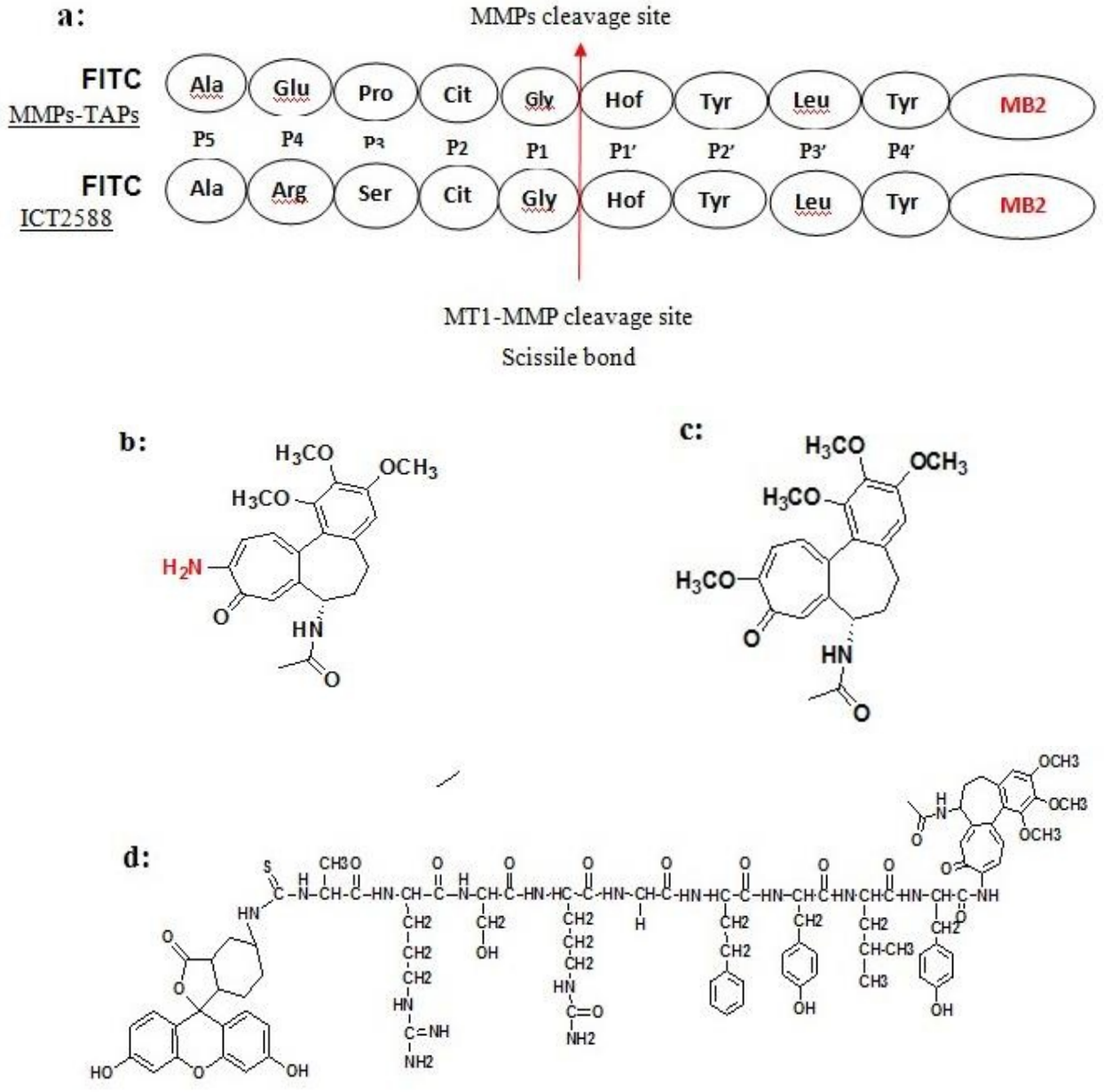

Figure 4. **(a)** peptide sequence of ICT2588. **(b)** chemical structure of azademethyl colchicines-MB2. **(c)** chemical structure of colchicines. **(d)** Peptide sequence of MMPs-TAPs and the chemical structure of ICT2588; MT1-MMP-targeted MB2 prodrug; chemical formula C96H117N17O23S, Exact mass 1907.82, Mol. Wt: 1909.12

2. Materials and Methods

Quantification of MMP expression by real-time reverse transcription-PCR

The 18S rRNA gene was used as an endogenous control and all the flu orogenic nucleotide probes were specific to MMPs-genes. The CT-cycle at which amplification entered the exponential phase was used as an indicator of the level of target RNA in each tissue, in which a lower CT indicated a higher quantity of target RNA. Cells were lysed on ice containing extraction buffer for 20 minutes. The resulting protein extract was collected via centrifugation at 1500 × g for 10minutes at 4°C. Equal amounts of proteins representing 1×10^5 cells were resolved by 10% SDS-PAGE and blotted onto Hybond-P membrane (Amersham, United Kingdom). The blot was probed with a monoclonal antibody to MT1-MMP (MAB3328; Chemi-Con International) overnight at 4°C.

Preparation of different cancer cell lines *in vitro*

The human fibro sarcoma (HT1080) and breast carcinoma (MCF-7) cell lines were selected according to their MT1-MMP expression profiles assessed by RT-PCR. HT1080 has been demonstrated to express high level of MT1-MMP. While MCF-7 in culture do not, table 2. Accordingly, HT1080 cell line is recommended as a positive control for MT1-MMP probing studies and MCF-7 cell lines as negative control (MT1-MMP negative). Cell lines were obtained from ECACC (Salisbury, Wiltshire, UK) and ATCC (Manassas, USA) were grown in vitro. All reagents and consumables were supplied by Sigma-Adrich Company Ltd (UK) unless otherwise specified.

All cell lines were cultivated in monolayer culture (T75 flasks) in complete Roswell Park Memorial Institute (RPMI) 1640 media containing 10% (v/v) fetal bovine serum (FBS) (Costar), 2 mmol/L L-glutamine and 1mmol/L sodium pyruvate. All cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. HT1080 and MCF-7 cell lines were regularly monitored for infection and allowed to grow to between 70-80% confluence after which they were washed in Hank's balanced salt solution (HBSS) and fresh media added as required. When sub-confluent, cells were harvested by trypsinization using 5ml 0.25% Trypsin/ethylenediaminetetra-acetic acid (EDTA) solution for 5 minutes at 37°C and centrifuged at 1000 rpm for 5 minutes at room temperature. Cells were either re-passaged in T75 flasks with 20ml of fresh media, or seeded into 96-well plate. For both cell lines, 2000 cells per well was selected as a seeding density for the cytotoxicity assays.

Assessment of MB2 and ICT2588 cytotoxicity *in vitro*

Cells were seeded in 96-well plates at 2000 cells per well in a total volume of 180µl RPMI media and allowed to adhere to the plate overnight (cells were seeded according to the standard operating procedure, SOP, of the cell culture laboratory/Institute of Cancer Therapeutics). Following 24-hour incubation, specific concentrations of the compounds of choice (MB2 and ICT258 dissolved in DMSO) were prepared by serial dilution using a fresh RPMI media. For MB2 and ICT2588, the serial concentrations were ranged from 10µM to 1nM (wide range). An aliquot volume (20µl) of all compounds was added to each lane (column) of the 96-well plates. In addition, one lane containing the same concentration of DMSO as the highest drug concentration (0.1% DMSO) was kept as a control. Each plate was set up for compounds exposure and incubated at 37°C for 72 hours after which the response of HT1080 and MCF-7 cell lines to MB2 and ICT2588 was investigated using the MTT assay. Having determined the IC_{50} values (concentration of drug required to reduce viable cell number by 50% relative to control) of MB2 and ICT2588-wide ranges, further narrow range concentrations of (20–100nM for MB2) and (0.01-10µM for ICT2588) were prepared and added to both cell lines in order to determine the exact IC_{50}.

Metabolism of ICT2588 in tumor xenografts and normal tissues

HT1080 and MCF7 tumor xenograft homogenates were used to study the *ex vivo* activation and stability of ICT2588 versus normal tissues. Xenograft tumors were homogenized by a specific activity buffer [50 mmol/ L Tris-HCl (pH7.6), 1.5 mmol/ L NaCl, 0.5 mmol/ L $CaCl_2$, 1 µmol/ L ZnCl, and 0.01% v/v Brij-35]. The end-product protein precipitate was analyzed by liquid chromatography-mass spectrometry (LC-MS). Recombinant MMPs (150 ng) were added to activate the prodrug ICT2588 (15 µm/L). Furthermore, the homogenates of MT1-MMP (+) HT1080 xenografts were incubated with ilomastat (GM6001), a broad spectrum MT-MMPs. Such strategy will help confirm the role of MMPs in the activation of ICT2588.

Studying the effect of P2′-amino acid substitution of the peptide sequence on the cleavage efficiency and selectivity of ICT2588 towards MT1-MMP

Since the ICT2588 is cleaved by MMP-2 at P2′-Tyr residue. Four derivatives of ICT2588 were obtained from the chemistry lab. (Dr. R. Falconer). Instead of Tyr, these prodrugs contain; Ser, Ala, Asp, and Asn amino acid residues at the $P^{2'}$ position. Similarly, wide range concentrations

(0.001 - 10μM) were prepared for each prodrug and added to 96-well plates of the MT1-MMP positive-HT1080 cell lines. Preparation of plates and drug additions were carried out in the same manner and under the same experimental conditions.

3. Results

Differential expression of MMP-1, -2, -3, -7, -8 and MT1-MMP in tumor models

Expression of mRNA of both MT1-MMP and MMPs is elevated in human cell lines grown in vitro. MT1-MMP were observed in the majority of cell lines analyzed, with extremes being the fibro sarcoma HT1080 and MDA231, (very high levels; CT ≤ 25). In contrast, the breast MCF7 cell lines showed almost negative results (Table 2).

Table 2 Quantification of MMP-mRNA expression by real-time reverse transcription-PCR in human cell lines grown *in vitro*. Classification of expression levels was determined from the CT of each gene as either very high (CT ≤ 25), high (CT = 26–30), moderate (CT = 31–35), low (CT = 36 39), or not detected (CT = 40).

Origin	cell lines	MMP1	MMP2	MMP3	MT1-MMP	MMP7	MMP8
Breast	MCF 7						
	MDA-231						
Colon	HT29						
	COLO205						
Fibrosarcoma	HT1080						
Prostate	PC3						
	LNCAP						

***In vitro* cytotoxicity profiles of MB2 and ICT2588 against MT1-MMP -positive and -negative cell lines:**

After 72 hr drug exposure, both HT1080 and MCF-7 cell lines were equally chemo sensitive to MB2, and IC_{50} values of MB2 are roughly the same in both cell lines (41 ±16 nmol/L for HT1080 and 60 ±12 for MCF7). Alternatively, *in vitro* cytotoxicity values of ICT2588 were as predicted. MT1-MMP expressing cell lines (TH1080 cell lines), was capable to cleave the prodrug ICT2588 and thus, showed lower IC_{50} values (180nmol/L ±35) compared to MCF-7 (MT1-MMP negative) (>7000 nmol/L; Table 3). Insensitivity of MCF-7 to ICT2588 means that the prodrug is still

inactive and unable to enter the cells (large and masked anticancer molecule) as a result of their negative expression profile of MT1-MMP.

Table 3 Differential *in vitro* cytotoxicity of the peptide-conjugate (ICT2588) and its warhead (MB2) in MT1-MMP-positive (HT1080) and –negative (MCF-7) cell lines. Cytotoxicity values are represented as the concentration required to reduce the viable cell number by 50% relative to control. All IC_{50} values were expressed as nmol/L.

Compound	HT1080 IC_{50} (nmol/L)	MCF-7 IC_{50} (nmol/L)
MB2	41 ± 16	60 ± 12
ICT2588	180 ± 35	> 7000

Activation of ICT2588 in tumor versus normal tissues and the effect of MT1-MMP blockade on its activity and metabolism

Metabolism and activation of ICT2588 were estimated *ex vivo* using HT1080 and MCF7 tumor xenografts and murine plasma and tissues. ICT2588 was relatively stable in MCF7 homogenate (t½ = 30.4 min). In contrast, extensive metabolism was seen in the HT1080 xenograft homogenate (t½ = 9.2 min). Murine plasma (t½ = 36 min) and liver (t½ = 27.5 min) (Figure 5).

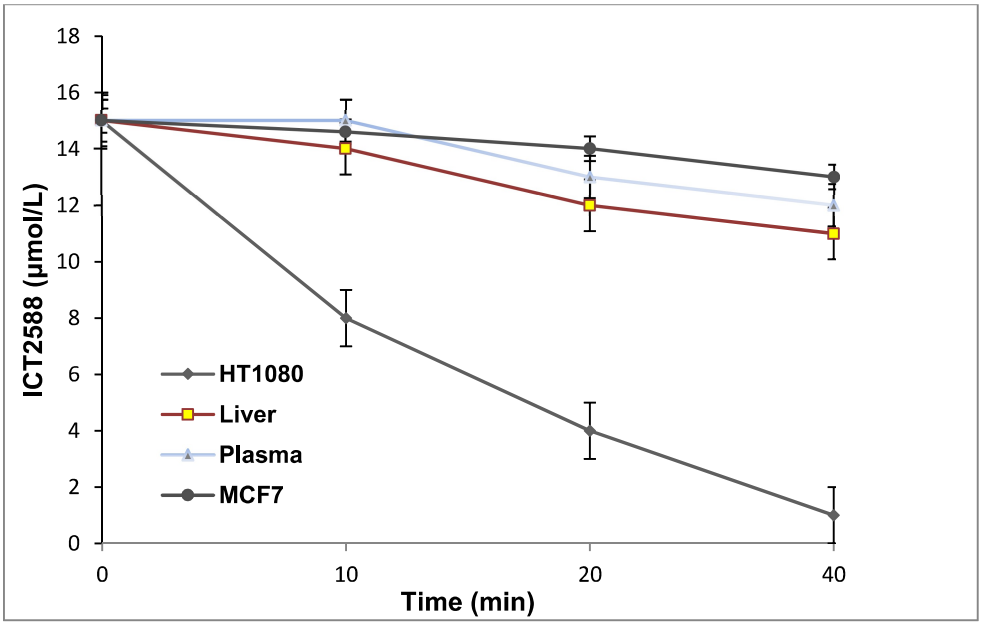

Figure 5. Activation of ICT2588 by MCF7 and HT1080 tumor xenografts in comparison to murine liver and plasma

The role of MMPs in the activation of ICT2588 was further confirmed in the presence of MT1-MMP inhibitor ilomastat (GM-6001). Ilomastat resulted in a dose-dependent decrease in the rate of ICT2588 activation by HT1080 (Figure 6).

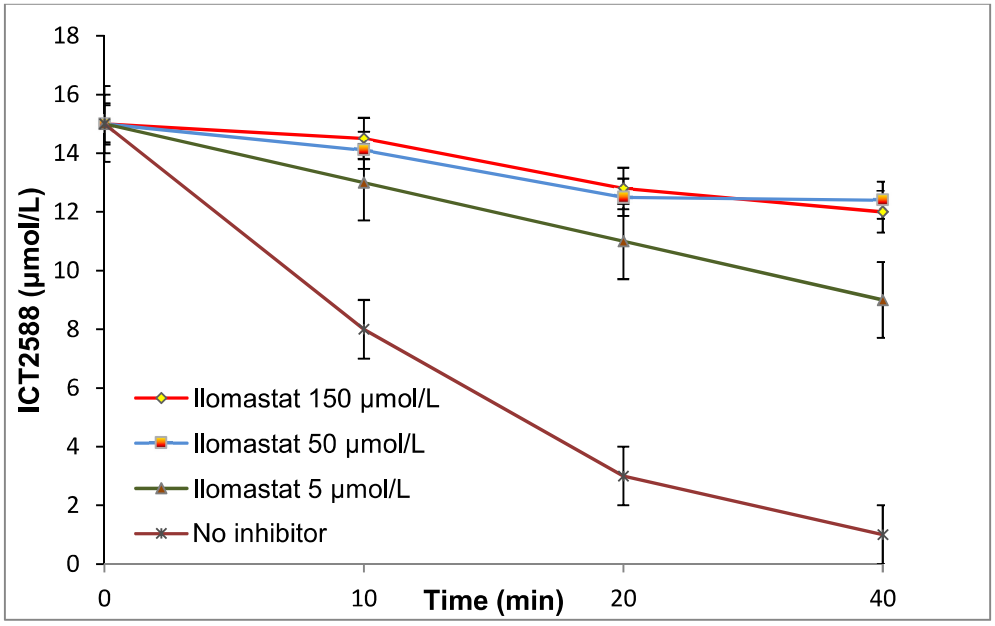

Figure 6. Rate of ICT2588 activation in the presence of serial concentrations of ilomastat (GM-6001)

Studying the effect of $P^{2'}$-amino acid substitution of the peptide sequence on the cleavage efficiency and selectivity of ICT2588 towards MT1-MMP:

Substitution of $P^{2'}$-Tyr with Alanine, Asparagine, Aspartic and Serine, was led to remarkable decrease in the cleavage efficiency of ICT2588 and thereby a decrease in its *in vitro* cytotoxicity against the MT1-MMP -expressive HT1080 (Figure 7).

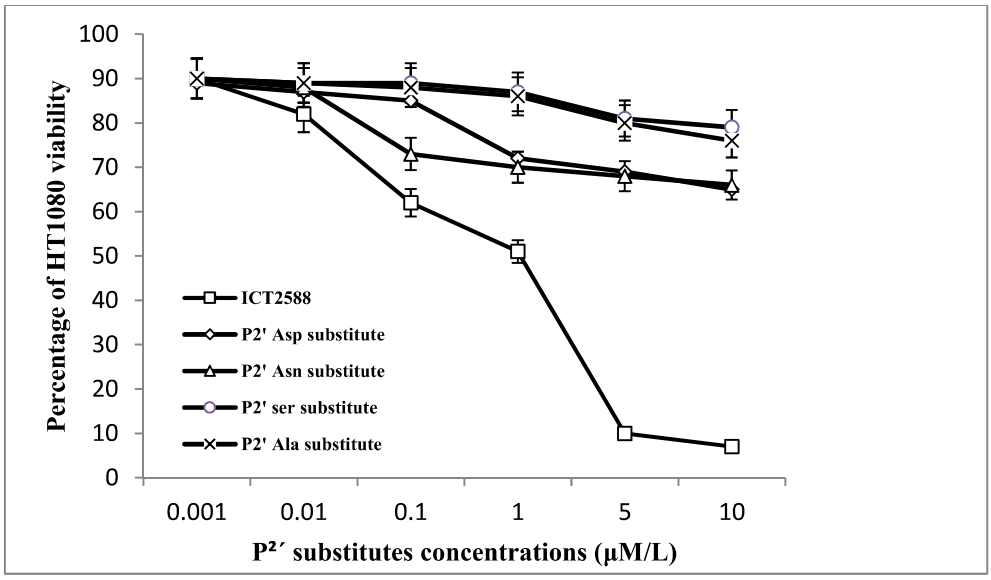

Figure 7. Effect of amino acid substitutions at P2'-position on the metabolism of peptide conjugate and thereby the liberation of the cytotoxic warhead-MB2

4. Discussion

Up to date, classical cytotoxic agents are unquestionably potent and still represent the basis of current treatment modalities. However, the clinical use of classical cytotoxics is limited by their off-target effects and lack of tumor-selective targeting. In this regard, most of the currently used anticancer agents have very narrow therapeutic index. One class of these potent chemotherapeutics is the vascular-disrupting agents "VDAs". Disruption of the established tumor vasculature is powerful chemotherapeutic strategy which has the effect of inducing tumor-blood vessel shutdown, nutrient and oxygen deprivation and consequently tumor necrosis. The ideal features of enzymes eligible for TAPs development are; elevated expression and activity in the tumor relative to normal tissues, high affinity for the pro-drug activation and potential for tumor development and/or progression. One enzyme class meets these features is the tumor-MMPs. The major limitation of secreted MMPs-activated pro-drugs is the innate ability of this enzyme class to travel small distances from tumor tissues which results in pro-drug activation in the normal tissues or leaking into the circulation. Accordingly, development of TAPs for activation by the membrane-type MMPs (MT-MMPs) is more attractive strategy as it would result in local and more specific pro-drug activation than the secreted MMPs-targeted TAPs. The research team at the Institute of Cancer Therapeutics has pre-clinically developed a novel TAP of VDA termed (ICT2588). This prodrug is designed for selective delivery of azademethyl-colchicine (MB2) to the tumor tissues by exploiting the cleavage activity of MT1-MMP in the tumor microenvironment. The prodrug is end-capped at its COOH terminus to prevent nonspecific exopeptidase degradation.

In this study, we showed the elevated expression profile of specific types-MMPs, particularly MT1-MMP in number of tumor types across a panel of selected preclinical tumor models. Activation of ICT2588 to its active warhead azademethyl-colchicine and differential *in vitro*

chemosensitivity in HT1080 (MT1-MMP positive) but not in the negative-MT1-MMP, MCF7 cancer cell lines. Selective cytotoxicity of ICT2588 was observed in the MT1-MMP positive HT1080 cell lines relative to MCF-7 cell line. These figures are supported by the increase in IC_{50} value from 180nM in HT1080 to more than 7μM in MCF-7 cell lines. In contrast, no differential cytotoxicity was demonstrated with MB2 (the active warhead alone) against HT1080 and MCF-7 cell lines, the IC_{50} values were 41nM and 60nM respectively. The change in IC_{50} values of ICT2588 support the rationale of MT1-MMP targeted prodrugs as illustrated by the inactivation of MB2-peptide conjugate and hence the lack of ICT2588 cytotoxicity in the MT1-MMP negative, MCF-7 cell line. Moreover, ICT2588 was selectively activated in HT1080 tumor xenografts but not by MCF7, murine and plasma tissues as they lack MT1-MMP activation machinery.

Since the aim of MT1-MMP-targeted therapeutics is to develop potent anticancer agents with better discrimination between tumor and normal cells and wider therapeutic index, taking the negative control MCF-7 cell lines as normal cells means that more than 7μM of ICT2588 is required to induce normal cell toxicity in comparison to 41nM of MB2 alone, and also the unique metabolism of ICT2588 by HT1080 tumor xenografts ($t_{½}$ = 9.2 min) *versus* ($t_{½}$ = 30.4 min) for MCF7 homogenate). These preliminary figures support the fact that the *in vitro* cytotoxic effect of MB2 alone was independent on the expression profile of MT1-MMP in both cell lines, as MB2 is a colchicine derivative induces cytotoxicity primarily by tubulin disruption. The small IC_{50} values of MB2 mean that the cleavage of ICT2588 will selectively release a very potent VDA at the tumor microenvironment. The distinctive role of Mt1-MMP in the activation of ICT2588 is further confirmed by the addition of IC_{50} doses of ICT2588 to different xenograft homogenates pre-treated with serial concentrations of MT-MMPs inhibitor ilomastat. However, the enzyme inhibitor "ilomastat" is non-selective inhibitor of MMPs and activation of ICT2588 by MMP-2 cannot be ruled out and another selective MMP-2 inhibitor, or knock out of MT1-MMP need to be performed in order to confirm the distinctive selectivity of ICT2588-peptide substrate towards MT1-MMP. As the prodrug "ICT2588" would selectively release a potent VDA (MB2), a peripheral viable rim of living cells at tumor boundaries is likely to remain following the use of ICT2588 as these cells receive oxygen and nutrient from the surrounding normal tissues rather than tumor vasculature. In this context, the use of ICT2588 would necessitate combination therapy with other anti-proliferative agents to target the viable rim. From the chemistry viewpoint, the peptide sequence of ICT2588 can accept further warhead moiety at their N-terminal residues. Accordingly, it might be possible to develop prodrugs with double warheads to target the tumor vasculature and the viable rim as well.

5. Conclusions

An increased understanding of the molecular basis of cancer has led to many developments in cancer management, which has now entered an era of molecularly-targeted therapeutics. TAPs are systemic entities that are selectively activated in tumor tissues by harnessing a unique physiological, metabolic or genetic differences between tumor and normal cells. The roles of end proteases in tumor development, expansion and metastasis is unequivocal, and accordingly, MT1-

MMP is ideal candidate for TAP development due to both its elevated activity in the extracellular tumor environment and its ability to selectively and specifically cleave short peptide sequences. Our lead compound in this study (ICT2588) has showed a remarkable result that might entitle the MT-MMPs as a cutting edge for development of novel anticancer prodrugs.

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